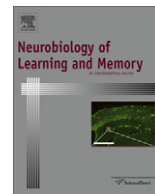




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Role of amygdala–prefrontal cortex circuitry in regulating the expression of contextual fear memory

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ABSTRACT

The basolateral amygdala (BLA) and medial prefrontal cortex (mPFC) are inter-connected regions involved in fear memory expression. The reciprocal nature of projections between these areas differs along the rostrocaudal extent of BLA. This study investigated the role of functional interactions between BLA and the prelimbic (PL) subregion of mPFC in mediating contextual fear memory. Freezing served as the measure of conditioned fear. Experiments 1–3 examined the effects of left, right or bilateral infusion of bupivacaine into anterior BLA (aBLA), posterior BLA (pBLA) or PL on fear memory expression. Reversible inactivation of left, right or bilateral aBLA impaired fear memory expression. Bilateral inactivation of pBLA or PL also disrupted the expression of fear memory, although left or right inactivation alone had no significant effects in either region. Experiment 4 examined the effects of functionally disconnecting pBLA and PL on contextual fear memory by infusing bupivacaine unilaterally into pBLA and PL in the ipsilateral or contralateral hemisphere. Fear memory expression was impaired by asymmetric inactivation of pBLA and PL; however, a similar effect was also observed with symmetric inactivation of these regions. Bupivacaine infusion did not affect behavior in the open field, likely ruling out non-specific effects of inactivation on innate fear and locomotor activity. These results demonstrate different roles for rostral and caudal BLA in mediating the expression of contextual fear memory. They also raise the possibility that pBLA–PL circuitry is involved in subserving fear memory expression via complex processing mechanisms, although further research is needed to confirm this preliminary finding.

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1. Introduction

Accumulating evidence indicates that Pavlovian fear learning and memory processing is mediated by neural circuits comprising reciprocally connected brain regions. Basolateral amygdala (BLA) and medial prefrontal cortex (mPFC) are two areas involved in the encoding and expression of learned fear (Lauzon & Laviolette, 2010; Pape & Paré, 2010). As BLA and mPFC share reciprocal projections which are functionally relevant (Laviolette, Lipski, & Grace, 2005; McDonald, 1991; McDonald, Mascagni, & Guo, 1996; Rosenkranz & Grace, 2002), interactions between these regions might subserve fear memory processing. Indeed, the BLA–mPFC circuit is involved in acquiring and consolidating learned fear. Blockade of cannabinoid transmission in the BLA–mPFC circuit impairs fear conditioning (Tan, Lauzon, Bishop, Bechard, & Laviolette, 2010). Synchronized neural activity between BLA and mPFC after fear learning predicts the consolidation of fear memory (Papa,

Duvarci, Popescu, Léna, & Paré, 2010). However, the role of BLA–mPFC circuitry in mediating fear memory expression remains unclear.

Previous studies have used functional disconnection to investigate how memory processing is regulated by interactions between BLA and mPFC (Fuchs, Eaddy, Su, & Bell, 2007; Mashhoon, Wells, & Kantak, 2010). In this procedure, BLA is inactivated in one hemisphere and mPFC is inactivated in the contralateral hemisphere. Any resulting deficits in memory processing are thought to be caused by perturbing communication within BLA–mPFC circuitry. The logic underpinning this strategy is that while unilateral inactivation leaves each area functionally intact, serial information flow between regions is disrupted by asymmetric inactivation. This assumes that BLA and mPFC share ipsilateral connections. However, the topographical organization of projections between these regions differs along the rostrocaudal extent of BLA. Whereas descending projections from the prelimbic (PL) subregion of mPFC target the anterior BLA (aBLA), the posterior BLA (pBLA) is the source of ascending projections to PL (Conde, Maire-Lepoivre, Audinat, & Crepel, 1995; Gabbott, Warner, Jays, Salway, & Busby, 2005; Kita & Kitai, 1990; McDonald, 1991; McDonald et al., 1996; Sesack, Deutch, Roth, & Bunney, 1989; Vertes, 2004). This anatomical specificity is also important given that rostral and

Abbreviations: aBLA, anterior basolateral amygdala; ANOVA, analysis of variance; BLA, basolateral amygdala; CeA, central amygdala; mPFC, medial prefrontal cortex; pBLA, posterior basolateral amygdala; PL, prelimbic cortex.

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caudal BLA may play different roles in mediating fear memory processing (Goossens & Maren, 2001; Scicli, Petrovich, Swanson, & Thompson, 2004).

Functional disconnection also assumes that unilateral inactivation of either region alone has no effect. However, evidence suggests that disrupting amygdala activity in only one hemisphere is sufficient to impair fear memory encoding and expression (Baker & Kim, 2004; LaBar & LeDoux, 1996). Moreover, the effects of unilateral PL inactivation on fear memory expression are unknown. This is crucial as an effect of unilateral inactivation in either region alone precludes the examination of functional disconnection. Also important to consider is the examination of unilateral inactivation of ipsilateral BLA and PL. While asymmetric inactivation effects are thought to represent disrupted serial processing within BLA–PL circuitry, any observed effects of symmetric inactivation may reflect the involvement of more complex processing mechanisms, such as parallel processing in this circuit or within a wider circuit comprising other inter-connected regions (Fuchs et al., 2007).

This study investigated the involvement of functional interactions between BLA and PL in mediating the expression of contextual fear memory. Although the hippocampus is well known for its role in contextual fear memory (Anagnostaras, Gale, & Fanselow, 2001), reversible inactivation studies show that BLA and PL are also involved in its expression (Corcoran & Quirk, 2007; Laurent & Westbrook, 2009; Maren & Holt, 2004; Muller, Corodimas, Fridel, & LeDoux, 1997). In Experiments 1–3, the sodium channel inhibitor bupivacaine was infused into aBLA, pBLA, or PL to examine the effects of reversibly inactivating these regions on fear memory expression. The effects of left, right or bilateral infusion of bupivacaine were determined separately as previous studies suggest that lateralized function of amygdala and mPFC is involved in fear memory processing (Baker & Kim, 2004; Fredrikson, Wik, Fischer, & Andersson, 1995; Hugdahl et al., 1995; Scicli et al., 2004). Based on the results of Experiments 1–3, the effects of functionally disconnecting pBLA and PL on fear memory expression were then determined in Experiment 4. The effects of unilateral infusion of bupivacaine into pBLA and PL in either the same or different hemisphere were examined. The effects of central bupivacaine infusion on behavior in the open field were also assessed to determine if any observed effects on fear memory expression were due to non-specific effects on innate fear and/or locomotor activity.

2. Materials and methods

2.1. Animals

Experiments were conducted in male Lister hooded rats (Charles River, UK) weighing 250–350 g at the time of surgery. Animals were housed on a 12-h light/dark cycle (lights on at 7 AM) with free access to food and water. Animals were group-housed before surgery. All experimental procedures were conducted with internal ethical approval and in accordance with the Animals (Scientific Procedures) Act 1986, UK. All behavioral testing occurred during the animals' light cycle.

2.2. Surgery

Anesthesia was induced with 3.5% isoflurane in a N₂O:O₂ mixture and maintained during surgery with 1.7–2.0% isoflurane to ensure complete inhibition of the hindpaw withdrawal reflex. Animals were placed in a stereotaxic frame and the incisor bar was adjusted to maintain the skull horizontal. Animals were implanted with guide cannulae (PlasticsOne, VA) 1 mm dorsal to the target injection site. In Experiments 1–3, animals were implanted with cannulae (26-gauge) bilaterally above aBLA, pBLA,

or PL. In Experiment 4, animals were implanted with cannulae unilaterally above pBLA and PL; cannulae were implanted above pBLA and the contralateral PL or above pBLA and the ipsilateral PL, with implants counter-balanced between the left and right hemispheres in the animals. The stereotaxic coordinates used in both experiments are as follows – aBLA: 2.5 mm posterior and 4.5 mm lateral to bregma, 6.4 mm ventral to the brain surface; pBLA: 3.2 mm posterior and 4.7 mm lateral to bregma, 6.6 mm ventral to the brain surface; PL: 3.2 mm anterior and 1.2 mm lateral (angled 12°) to bregma, 2 mm ventral to the brain surface (Paxinos & Watson, 1998). Cannulae were secured with dental cement to 3–4 screws threaded into the skull. Obturators (33-gauge; PlasticsOne) that extended 1 mm beyond the tip of the guide cannulae were fitted immediately after implantation. Animals were singly housed after surgery. From 1 to 2 days after surgery, animals were subjected daily to mild restraint during which time the obturators were loosened and re-tightened. This ensured that the cannulae remained unblocked after surgery and also served to habituate the animals to handling during the central drug infusion procedure. Behavioral testing commenced 5–7 days after surgery.

2.3. Central drug infusions

Bupivacaine hydrochloride (Sigma, US) was dissolved in saline to a concentration of 0.75% w/v, calculated as the salt of the drug. This concentration has been used previously to examine the effects of BLA or PL inactivation on behavior (McLaughlin & Floresco, 2007; Floresco, Block, & Tse, 2008). Bupivacaine and/or vehicle (saline) were infused in a volume of 0.5 μ L over 1 min via injector cannulae (33-gauge; PlasticsOne) extending 1 mm beyond the tip of the guide cannulae connected to 1 μ L syringes by a length of polyethylene tubing. In Experiments 1–3, different groups of animals received one of the following central drug infusions into aBLA, pBLA or PL: (1) bilateral vehicle, (2) left bupivacaine/right vehicle, (3) right bupivacaine/left vehicle, or (4) bilateral bupivacaine. In Experiment 4, three groups received one of the following drug infusions unilaterally into both pBLA and PL: (1) vehicle in pBLA and contralateral PL, (2) bupivacaine in pBLA and ipsilateral PL, or (3) bupivacaine in pBLA and contralateral PL. Injectors were left in place for 1 min following infusions before they were removed and the obturators replaced. Behavioral testing commenced 10 min later to minimize the diffusion of drug beyond the site of infusion.

2.4. Contextual fear conditioning and memory testing

The acquisition of contextual fear conditioning and the expression of contextual fear memory were assessed using a 2-day testing paradigm. The apparatus used has been described in detail elsewhere (Stevenson, Spicer, Mason, & Marsden, 2009). On the first day, each animal was conditioned in a novel context consisting of distinct visual (black and white stripes on two walls of the chamber), auditory (60 dB white noise) and olfactory (20% ethanol; see below) cues present throughout testing. The unconditioned stimulus used was mild electric shock (0.5 mA, 1 s duration) delivered through the floor bars of the chamber. Shock delivery was controlled automatically by computer (MED-PC IV software, Med Associates, VT). The animal was placed in the chamber and after 2 min was subjected to six unsignalled shocks (2 min inter-trial interval). The animal was removed from the chamber 2 min after the last footshock and returned to the home cage. Animals received no central drug infusions on the first day. On the second day, the effects of aBLA, pBLA, or PL inactivation (Experiments 1–3) or pBLA–PL functional disconnection (Experiment 4) on fear memory expression were tested. Each animal received central drug infusions before being returned to the conditioning context for

5 min. Behavior on both days was recorded using a digital camera positioned above the chamber for subsequent data analysis. Floor bars and waste tray were cleaned with 20% ethanol between each session. Animals were tested at approximately the same time of day on both days.

2.5. Open field testing

The effects of aBLA, pBLA or PL inactivation (Experiments 1–3) or pBLA–PL functional disconnection (Experiment 4) on innate fear and locomotor activity in the open field were also examined. The same animals used in the fear memory expression experiments were used for open field testing (Corcoran & Quirk, 2007; Sierra-Mercado, Padilla-Coreano & Quirk, 2011). Animals were tested 3–7 days after the fear memory test. The apparatus and testing procedures used have been described in detail elsewhere (Stevenson, Meredith, Spicer, Mason, & Marsden, 2009). Following central drug infusion (see above), each animal was placed in the open field for 10 min. Behavior was digitally recorded for subsequent data analysis.

2.6. Histology

After completing the experiments animals were humanely culled with carbon dioxide (Floresco & Ghods-Sharifi, 2007). The brains were removed and post-fixed in 4% paraformaldehyde kept at 4 °C until sliced. The brains were sliced and BLA sections were stained for acetylcholinesterase as previously described (Stevenson, Halliday, Marsden, & Mason, 2007).

2.7. Data analysis

Freezing, defined as the absence of movement except for that related to respiration, was taken as the behavioral measure of fear during contextual conditioning and memory testing. Freezing was scored manually and assessed blind to the treatment group of each animal. During conditioning, freezing was assessed at 3 s intervals before the first and after the last footshock presentation. The cumulative duration of freezing was then calculated and expressed as a percentage of both 2 min durations (i.e. before and after conditioning). Differences in freezing before and after conditioning between the groups to receive different drug infusions the next day were analyzed using a two-way analysis of variance (ANOVA). The between-subject factor was group and the within-subject factor was time (i.e. before and after conditioning). During fear memory testing the following day, freezing was determined at 3 s intervals throughout the 5 min session and determined as above for conditioning. Differences in freezing between the drug treatment groups during fear memory testing were analyzed in two ways. Freezing over the entire 5 min session was analyzed using a one-way ANOVA, with group as the between-subject factor. Freezing during each 1-min bin of the test session was also analyzed separately using a two-way ANOVA. The between-subject factor was group and the within-subject factor was time. Digitally recorded behavior in the open field was analyzed using Ethovision software (Noldus, Netherlands). The duration of time spent in the center and the total distance moved in the open field were determined and taken as indices of innate fear and locomotor activity, respectively. Differences between the treatment groups on these behavioral measures were analyzed separately using one-way ANOVA, with group as the between-subject factor. All post hoc comparisons were conducted using the Tukey's test. All data are presented as the mean + SEM. The level of significance for all comparisons was set at $P < 0.05$.

3. Results

Only data from animals with histologically confirmed cannulae implanted bilaterally into aBLA, pBLA or PL were included in the analysis for Experiments 1–3 (Fig. 1A–C). Only data from animals with histologically confirmed cannulae implanted unilaterally into pBLA and PL were included in the analysis for Experiment 4 (Fig. 1D). A total of 118 animals ($n = 7–9/\text{group}$) in Experiments 1–4 met histological criteria for inclusion in the data analysis.

3.1. Expt 1: Unilateral and bilateral inactivation of aBLA impair fear memory expression

The effects of aBLA inactivation on the expression of contextual fear memory are shown in Fig. 2. Animals were subjected to fear conditioning without drug (Fig. 2A) and the next day received intra-aBLA drug infusions before testing fear memory in the conditioning context (Fig. 2B and C). Analysis of freezing behavior immediately before and after conditioning revealed a significant main effect of time ($F_{(1,26)} = 309.3$, $P < 0.0001$) but no main effect of group ($F_{(3,26)} = 0.60$, $P > 0.05$) or time \times group interaction ($F_{(3,26)} = 0.35$, $P > 0.05$). Whereas the animals showed negligible freezing before conditioning, they did exhibit freezing after conditioning. Post-hoc analysis indicated that freezing was significantly increased after conditioning ($P < 0.0001$). No significant differences in freezing were observed between the groups ($P > 0.05$) to receive different drug treatments the following day (Fig. 2A).

Before assessing fear memory, different groups of animals received one of the following drug infusions into aBLA: (1) vehicle, (2) left bupivacaine, (3) right bupivacaine, or (4) bilateral bupivacaine. Analysis of freezing during the entire fear memory test session after drug infusion revealed a significant main effect of group ($F_{(3,26)} = 9.56$, $P < 0.001$). Post-hoc analysis indicated that freezing was significantly decreased with bilateral bupivacaine infusion, compared to vehicle ($P < 0.01$), showing that bilateral inactivation impaired fear memory expression. Infusions of bupivacaine into the left or right hemisphere alone also caused a significant decrease in freezing ($P < 0.01$), indicating that unilateral aBLA inactivation was sufficient to disrupt the expression of fear memory (Fig. 2B).

A more detailed analysis of the time course of freezing during fear memory testing is presented in Fig. 2C. The analysis revealed significant main effects of time ($F_{(4,26)} = 5.43$, $P < 0.001$) and group ($F_{(3,26)} = 8.99$, $P < 0.001$) but no time \times group interaction ($F_{(12,104)} = 0.96$, $P > 0.05$). Post-hoc analysis indicated that freezing was significantly greater at 3–4 min, compared to 1 min ($P < 0.01$). Moreover, compared to vehicle, freezing was significantly decreased by left, right or bilateral bupivacaine infusion ($P < 0.05$), confirming the results for freezing during the test session as a whole.

The effects of aBLA inactivation on behavior in the open field are shown in Table 1. Separate analyses revealed no significant main effect of group for time spent in the center ($F_{(3,26)} = 0.49$, $P > 0.05$) or total distance moved ($F_{(3,26)} = 0.24$, $P > 0.05$) in the open field. This indicates that inactivation had no effect on innate fear or locomotor activity.

3.2. Expt 2: Bilateral, but not unilateral, pBLA inactivation impairs fear memory expression

The effects of pBLA inactivation on fear memory expression are shown in Fig. 3. Animals were tested drug-free during conditioning (Fig. 3A) and received drug infusions as above into pBLA before testing fear memory the next day (Fig. 3B and C). Analysis of freezing behavior before and after conditioning revealed a significant

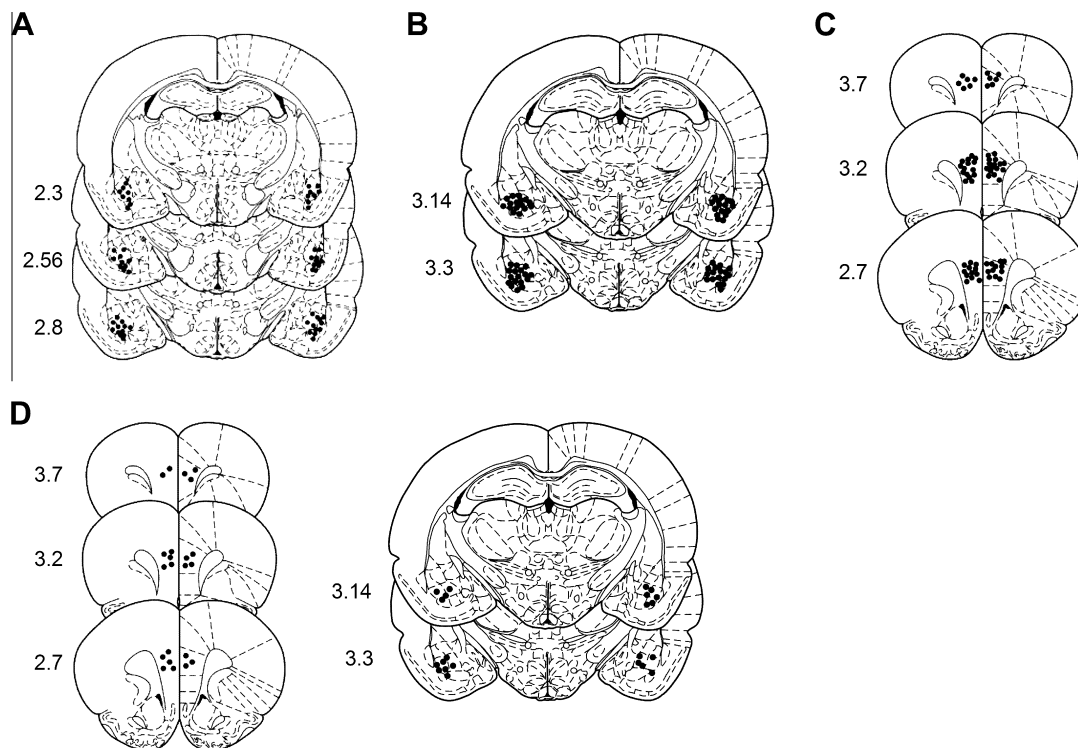


Fig. 1. Schematic representation of cannula placements in (A) aBLA, (B) pBLA, and (C) PL in Experiments 1–3, respectively. (D) Cannula placements in pBLA and PL in Experiment 4. Numbers beside BLA and PL sections indicate distance (mm) posterior and anterior, respectively, to bregma (adapted from Paxinos and Watson (1998)).

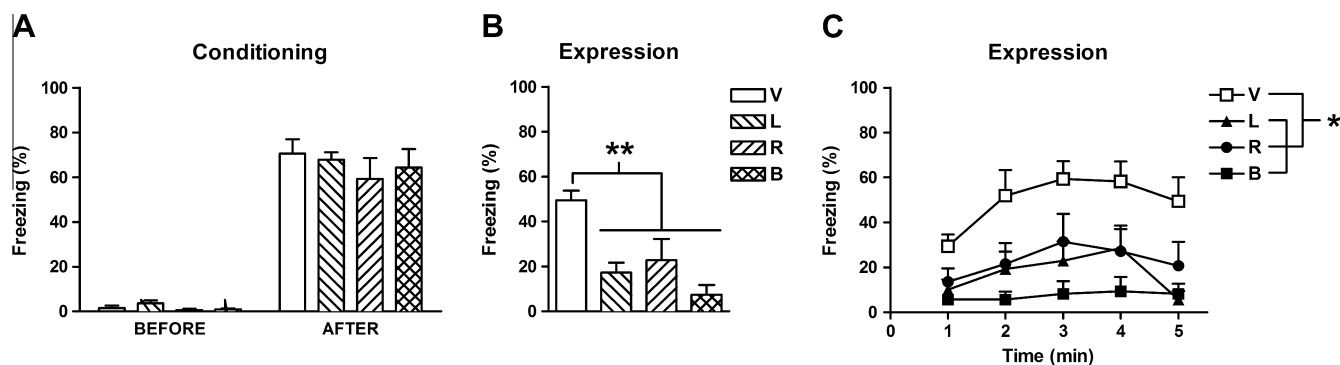


Fig. 2. Effects of bupivacaine infusion into aBLA on the expression of contextual fear memory. (A) Freezing before and after shock presentations in the groups to receive different drug infusions the next day. No differences in freezing were observed between the groups on the day of conditioning. (B) Freezing during the fear memory test the following day. Prior to memory testing, animals received infusions of vehicle (V), bupivacaine into the left (L) or right (R) hemisphere alone, or bilateral (B) bupivacaine. Compared to V, freezing was significantly decreased with L, R or B (** $P < 0.01$). (C) Time course of freezing for each 1 min bin during the test session. Again, freezing was significantly decreased by L, R, or B, compared to V (* $P < 0.05$).

main effect of time ($F_{(1,29)} = 241.7$, $P < 0.0001$) but no main effect of group ($F_{(3,29)} = 0.92$, $P > 0.05$) or time \times group interaction ($F_{(3,29)} = 0.56$, $P > 0.05$). Post-hoc analysis indicated that freezing was significantly increased after conditioning ($P < 0.0001$). There were no significant differences in freezing between the groups ($P > 0.05$) to receive different drug treatments the following day (Fig. 3A).

Analysis of freezing over the whole fear memory test after intra-pBLA drug infusion revealed a significant main effect of group ($F_{(3,29)} = 5.39$, $P < 0.01$). Post-hoc analysis indicated that freezing was significantly decreased with bilateral bupivacaine infusion, compared to vehicle ($P < 0.01$), showing that bilateral inactivation impaired fear memory expression. Decreases in freezing were also observed with left or right infusion of bupivacaine. However, these

effects were not significant ($P > 0.05$), indicating that unilateral inactivation had no effect on fear memory expression (Fig. 3B).

This was confirmed by the time course analysis of freezing during fear memory testing (Fig. 3C). The analysis revealed significant main effects of time ($F_{(4,29)} = 20.85$, $P < 0.0001$) and group ($F_{(3,29)} = 5.19$, $P < 0.01$) and a significant time \times group interaction ($F_{(12,116)} = 3.02$, $P < 0.01$). Post-hoc analysis indicated that, compared to 1 min, freezing was significantly increased at 3–4 min with infusion of vehicle, left bupivacaine, or right bupivacaine ($P < 0.01$), but not bilateral bupivacaine ($P > 0.05$). Furthermore, bilateral infusion of bupivacaine significantly decreased freezing at 2–4 min, compared to vehicle ($P < 0.01$). Although freezing was also decreased by left or right infusion of bupivacaine, these effects were not significant at any time ($P > 0.05$).

The effects of pBLA inactivation on open field behavior are shown in Table 1. Analyses of time spent in the center and total distance moved in the open field revealed no significant main effect of group (center: $F_{(3,29)} = 0.99$, $P > 0.05$; distance: $F_{(3,29)} = 0.05$, $P > 0.05$), indicating that inactivation had no effect on innate fear or locomotor activity.

3.3. Expt 3: Bilateral, but not unilateral, inactivation of PL impairs fear memory expression

The effects of PL inactivation on fear memory expression are presented in Fig. 4. Animals were drug-naïve during conditioning (Fig. 4A) and the next day were infused with drug as above into PL before testing fear memory (Fig. 4B and C). Analysis of freezing behavior before and after conditioning revealed a significant main effect of time ($F_{(1,29)} = 190.9$, $P < 0.0001$) but no main effect of group ($F_{(3,29)} = 0.79$, $P > 0.05$) or time \times group interaction ($F_{(3,29)} = 0.76$, $P > 0.05$). Post-hoc analysis indicated that freezing was significantly greater after ($P < 0.0001$), compared to before, conditioning. There were no significant differences in freezing between any of the groups ($P > 0.05$) to receive different drug treatments the next day (Fig. 4A).

Analysis of freezing during the entire fear memory test revealed a significant main effect of group ($F_{(3,29)} = 3.00$, $P < 0.05$). Post-hoc analysis indicated that bupivacaine infused bilaterally caused a significant decrease in freezing, compared to vehicle ($P < 0.05$), showing that bilateral inactivation disrupted the expression of fear

memory. Although decreases in freezing were also observed with left or right bupivacaine infusion, these effects were not significant ($P > 0.05$). This indicates that unilateral PL inactivation did not affect fear memory expression (Fig. 4B).

The time course of freezing during the test session is presented in Fig. 4C. The analysis revealed significant main effects of time ($F_{(4,29)} = 11.02$, $P < 0.0001$) and group ($F_{(3,29)} = 2.91$, $P = 0.05$) but no time \times group interaction ($F_{(12,116)} = 0.83$, $P > 0.05$). Post-hoc analysis indicated that freezing was significantly greater at 2–5 min, compared to 1 min ($P < 0.05$). Moreover, bilateral bupivacaine infusion significantly decreased freezing, compared to vehicle ($P < 0.05$). Freezing was also decreased by left or right infusion of bupivacaine, although not significantly so ($P > 0.05$). This confirmed the results for freezing during the whole test session.

The effects of PL inactivation on behavior in the open field are shown in Table 1. Separate analyses revealed no significant main effect of group for time spent in the center ($F_{(3,29)} = 0.48$, $P > 0.05$) or total distance moved ($F_{(3,29)} = 1.51$, $P > 0.05$) in the open field. This indicates that inactivation had no effect on innate fear or locomotor activity.

3.4. Expt 4: Asymmetric and symmetric pBLA–PL inactivation impair fear memory expression

Experiment 1 showed that the expression of fear memory was impaired by unilateral inactivation of aBLA. This precludes

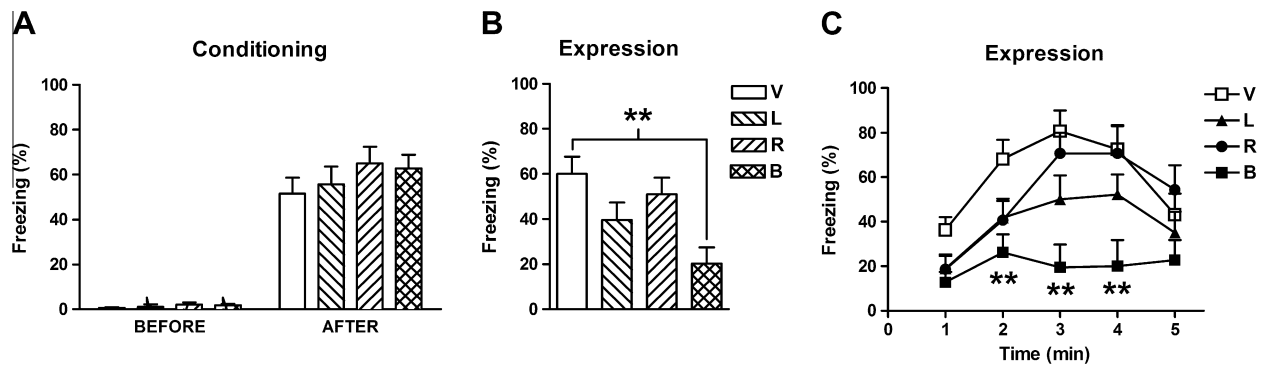


Fig. 3. Effects of intra-pBLA infusion of bupivacaine on fear memory expression. (A) There were no differences in freezing between the groups before or after conditioning. (B) Freezing during fear memory testing. Compared to vehicle (V), bilateral (B) infusion of bupivacaine significantly decreased freezing (** $P < 0.01$). There were no significant effects of left (L) or right (R) bupivacaine infusion. (C) Time course of freezing during the test session. Again, B significantly decreased freezing, compared to V (** $P < 0.01$), whereas L or R had no significant effects.

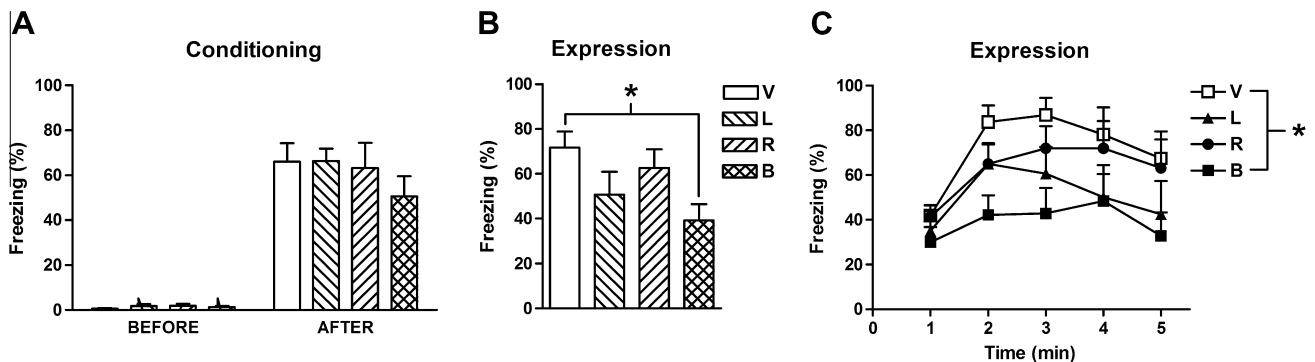


Fig. 4. Effects of intra-PL bupivacaine infusion on fear memory expression. (A) There were no differences in freezing between the groups before or after conditioning. (B) Freezing during fear memory testing. Compared to vehicle (V), bilateral (B) bupivacaine infusion significantly decreased freezing (* $P < 0.05$). There were no significant effects of left (L) or right (R) bupivacaine infusion on freezing. (C) Time course of freezing during the test session. Again, B significantly decreased freezing, compared to V (* $P < 0.05$), whereas L or R had no significant effects.

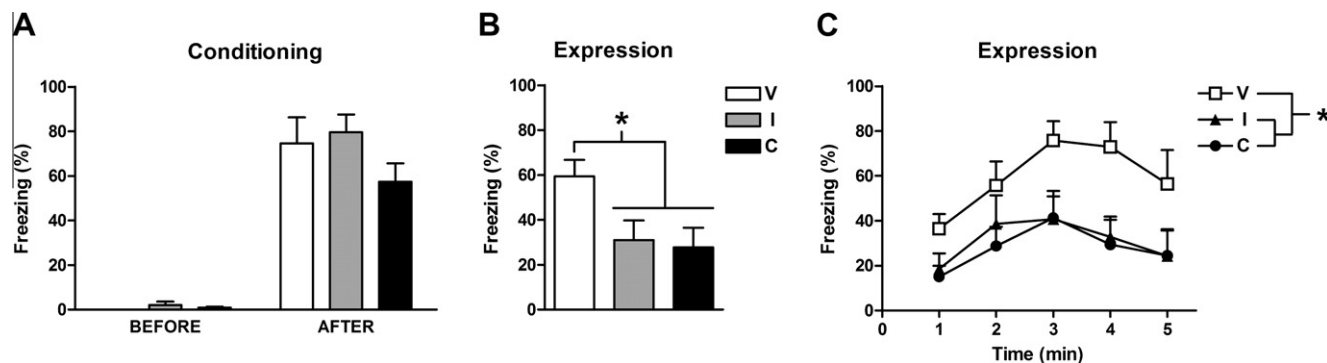


Fig. 5. Effects of unilateral infusion of bupivacaine into pBLA and either the ipsilateral (I) or contralateral (C) PL on fear memory expression. (A) There were no differences in freezing between the groups before or after conditioning. (B) Freezing during fear memory testing. Compared to vehicle (V), both C and I significantly decreased freezing ($*P < 0.05$). (C) Time course of freezing during the test session. Again, C and I both significantly decreased freezing, compared to V ($*P < 0.05$).

examining the effects of functional disconnection of aBLA and PL on fear memory expression. Experiments 2 and 3 showed that unilateral inactivation of either pBLA or PL alone had no significant effects on the expression of learned fear. Thus Experiment 4 examined the effects of functionally disconnecting pBLA and PL on fear memory expression.

The effects of unilateral pBLA and PL inactivation in either the ipsilateral or contralateral hemisphere on fear memory expression are shown in Fig. 5. Animals were conditioned drug-free (Fig. 5A) and received contralateral vehicle, ipsilateral bupivacaine, or contralateral bupivacaine infusions before memory testing the next day (Fig. 5B and C). Analysis of freezing behavior before and after conditioning revealed a significant main effect of time ($F_{(1,19)} = 172.0$, $P < 0.0001$) but no main effect of group ($F_{(2,19)} = 1.66$, $P > 0.05$) or time \times group interaction ($F_{(2,19)} = 1.63$, $P > 0.05$). Post-hoc analysis indicated that freezing was significantly greater after, compared to before, conditioning ($P < 0.0001$). There were no significant differences in freezing between the groups ($P > 0.05$) to receive different drug treatments the next day (Fig. 5A).

Analysis of freezing during the fear memory test in its entirety revealed a significant main effect of group ($F_{(2,19)} = 4.29$, $P < 0.05$). Post-hoc analysis indicated that unilateral bupivacaine infused

into contralateral pBLA and PL significantly decreased freezing, compared to vehicle ($P < 0.05$). This indicates that asymmetric inactivation of pBLA and PL impairs fear memory expression. However, unilateral bupivacaine infusion into ipsilateral pBLA and PL also induced a significant decrease in freezing ($P < 0.05$), suggesting that symmetric inactivation of these regions is sufficient to disrupt fear memory expression (Fig. 5B).

The time course of freezing during fear memory testing is presented in Fig. 5C. The analysis revealed significant main effects of time ($F_{(4,19)} = 7.52$, $P < 0.0001$) and group ($F_{(2,19)} = 4.34$, $P < 0.05$) but no time \times group interaction ($F_{(8,76)} = 0.67$, $P > 0.05$). Post-hoc analysis indicated that freezing was significantly greater at 2–4 min, compared to 1 min ($P < 0.05$). Furthermore, compared to vehicle, unilateral bupivacaine infusion into contralateral or ipsilateral pBLA and PL significantly decreased freezing ($P < 0.05$), confirming the results for freezing during the test session as a whole.

The effects of unilateral pBLA and PL inactivation in the ipsilateral or contralateral hemisphere on open field behavior are shown in Table 1. Analyses of time spent in the center and total distance moved in the open field revealed no significant main effect of group (center: $F_{(2,17)} = 0.34$, $P > 0.05$; distance: $F_{(2,17)} = 0.13$, $P > 0.05$), indicating that symmetric or asymmetric inactivation of pBLA and PL had no effects on innate fear or locomotor activity.

Table 1

There were no effects of left, right or bilateral infusion of bupivacaine into aBLA, pBLA or PL on time spent in the center or distance moved in the open field. There were also no effects of unilateral bupivacaine infusion into pBLA and the ipsilateral or contralateral PL on time spent in the center or distance moved in the open field. Thus bupivacaine had no effect on innate fear or locomotor activity.

	Time in center (%)	Distance moved (cm)
aBLA inactivation		
Vehicle	24.1 \pm 3.5	3704.6 \pm 109.5
Left	27.4 \pm 2.3	3981.4 \pm 366.0
Right	22.7 \pm 3.4	3719.2 \pm 268.5
Bilateral	23.0 \pm 2.9	3826.6 \pm 205.2
pBLA inactivation		
Vehicle	24.9 \pm 4.6	3546.4 \pm 202.0
Left	26.3 \pm 3.3	3459.1 \pm 166.0
Right	18.9 \pm 4.0	3386.0 \pm 335.5
Bilateral	28.3 \pm 2.6	3443.7 \pm 533.4
PL inactivation		
Vehicle	25.5 \pm 3.8	3564.2 \pm 242.9
Left	22.3 \pm 4.6	3027.8 \pm 123.9
Right	20.2 \pm 2.4	3097.3 \pm 230.2
Bilateral	23.9 \pm 1.9	3124.4 \pm 169.4
pBLA/PL disconnection		
Vehicle	24.7 \pm 5.1	3260.9 \pm 334.2
Ipsilateral	28.9 \pm 3.3	3448.6 \pm 266.1
Contralateral	26.6 \pm 2.2	3402.9 \pm 193.7

4. Discussion

This study investigated the role of functional interactions between BLA and PL in mediating contextual fear memory. Experiments 1–3 showed that bilateral inactivation of BLA or PL impaired the expression of fear memory. In Experiment 1, unilateral inactivation of aBLA disrupted fear memory expression to the same extent as bilateral inactivation. There was no evidence of lateralization as inactivation of left or right aBLA had similar effects. Unilateral inactivation of pBLA (Experiment 2) or PL (Experiment 3) had no significant effects on fear memory expression, again demonstrating a lack of lateralization. Experiment 4 showed that asymmetric inactivation of pBLA and PL impaired the expression of fear memory; however, symmetric pBLA–PL inactivation had a similar effect. There were no effects of inactivation on behavior in the open field, indicating that impaired fear memory expression was not likely attributable to non-specific effects on innate fear or locomotor activity. These results demonstrate that rostral and caudal BLA play different roles in mediating the expression of contextual fear memory. They also raise the possibility that functional interactions between pBLA and PL are involved in subserving fear memory expression.

4.1. Roles of aBLA, pBLA and PL in fear memory expression

The finding of impaired expression of contextual fear memory with reversible inactivation of BLA is in agreement with other studies (Maren & Holt, 2004; Muller et al., 1997). Here, the effects of inactivating aBLA or pBLA were examined separately as the reciprocal nature of connections between BLA and PL differs along the rostrocaudal extent of BLA. While PL sends descending projections to aBLA, ascending projections to PL arise from pBLA. Moreover, whereas projections from pBLA to PL are predominantly ipsilateral, PL projects bilaterally to aBLA (Conde et al., 1995; Gabbott et al., 2005; Kita & Kitai, 1990; McDonald, 1991; McDonald et al., 1996; Sesack et al., 1989; Vertes, 2004). Thus the effects of functionally disconnecting BLA and PL might be expected to differ with inactivation of rostral or caudal BLA (e.g. Floresco & Ghods-Sharifi, 2007; Fuchs et al., 2007). This is also important given the evidence for rostral BLA being preferentially involved in fear memory expression. Increased Fos expression has been shown in aBLA, but not pBLA, after testing contextual fear memory (Scicli et al., 2004). Although the present results also suggest that rostral and caudal BLA play different roles in mediating fear memory expression, they are at odds with this previous study. Here, bilateral, but not unilateral, inactivation of pBLA impaired fear memory expression, supporting a role for caudal BLA in subserving contextual fear memory. In contrast, fear memory expression was disrupted to a similar extent by unilateral or bilateral inactivation of aBLA. There was also no evidence of lateralized BLA function which contradicts other studies showing a preferential role for right amygdala in contextual fear memory processing (Baker & Kim, 2004; Goosens & Maren, 2001; Scicli et al., 2004). Differences between studies in the animal strain or experimental procedures used may account for these apparent discrepancies.

Although different roles for rostral and caudal BLA in memory mechanisms have been shown previously (Kantak, Black, Valencia, Green-Jordan, & Eichenbaum, 2002; McLaughlin & Floresco, 2007), it is unclear why differences in the hemispheric nature of fear memory processing were observed here. One possibility is that bupivacaine infused into aBLA diffused to the adjacent central nucleus of the amygdala (CeA), another amygdaloid subregion crucial for mediating the expression of learned fear (Pape & Paré, 2010). A previous study showed that large unilateral amygdala lesions, which included damage to both BLA and CeA, given post-training were sufficient to impair the expression of contextual fear memory (Baker & Kim, 2004). However, a recent study examining the effects of more selective damage to BLA and CeA found no effects of unilateral lesions on contextual fear memory (Jimenez & Maren, 2009). Moreover, in the present study no effects of aBLA or pBLA inactivation were observed on innate fear in the open field. Evidence indicates that inactivation of CeA, but not BLA, reduces unconditioned fear (Carvalho, Moreira, Zanoveli, & Brandão, 2010; Moreira, Masson, Carvalho, & Brandão, 2007), suggesting that there was no appreciable diffusion of bupivacaine into CeA here. It is also possible that the necessity of activating one or both hemispheres of aBLA or pBLA, respectively, for mediating fear memory processing is due to differences in the inter-hemispheric connectivity of rostral and caudal BLA. Whereas left and right aBLA share strong reciprocal projections, the connections between left and right pBLA are relatively sparse (Savander, Ledoux, & Pitkänen, 1997). Interestingly, unilateral inactivation of BLA was recently shown to be sufficient for impairing the acquisition of conditioned defeat. Furthermore, unilateral BLA inactivation attenuated Fos expression in the contralateral hemisphere induced by conditioned defeat, suggesting that unilateral activation evokes activity contralaterally (Markham, Taylor, & Huhman, 2010). Similar inter-hemispheric processing mechanisms in rostral BLA could be involved in mediating fear memory expression. However, this line of reasoning

is difficult to reconcile with the lack of effect observed with unilateral PL inactivation on the expression of learned fear, given the robust inter-hemispheric connectivity of mPFC (Carr & Sesack, 1998).

The finding of impaired fear memory expression with reversible PL inactivation agrees with previous studies (Corcoran & Quirk, 2007; Laurent & Westbrook, 2009). It should be noted that this effect could possibly have resulted from bupivacaine diffusing into neighboring mPFC subregions. Indeed, reduced fear memory expression has been shown with combined inactivation of PL and infralimbic cortex (Sierra-Mercado, Corcoran, Lebrón-Milad, & Quirk, 2006). However, more recent studies have shown that inactivation of PL, but not infralimbic cortex, reduces fear memory expression (Laurent & Westbrook, 2009; Sierra-Mercado et al., 2011). It is also possible that bupivacaine diffusion into anterior cingulate cortex contributed to the effects ascribed to PL inactivation. This would also appear unlikely though given the lack of effects of inactivating this area on the expression of recent fear memory (Frankland, Bontempi, Talton, Kaczmarek, & Silva, 2004). In the present study there was no evidence of lateralized PL function. This suggests that bupivacaine infused into one hemisphere of PL did not diffuse appreciably into the contralateral hemisphere, an important consideration for functional disconnection experiments entailing unilateral drug infusion into mPFC. Other studies have also shown no effects of unilateral mPFC inactivation on behavior (Floresco & Ghods-Sharifi, 2007; Fuchs et al., 2007; Mashhoon et al., 2010). The present study found no effects of PL inactivation on behavior in the open field, replicating previous findings (Corcoran & Quirk, 2007; Sierra-Mercado et al., 2011).

4.2. Role of BLA–PL circuitry in fear memory expression

The results of Experiments 1–3 provided the basis for investigating the role of functional interactions between BLA and PL in subserving the expression of fear memory. Given that unilateral aBLA inactivation impaired fear memory expression, the effects of functionally disconnecting rostral BLA and PL were not examined. As no significant effects of unilateral inactivation of pBLA or PL were observed, functional disconnection of these regions was examined. Unilateral inactivation of pBLA and contralateral PL disrupted the expression of fear memory. Taken on its own, this finding would suggest that serial processing in pBLA–PL circuitry is involved in fear memory expression. However, unilateral inactivation of ipsilateral pBLA and PL had the same effect. Similar impairments with asymmetric and symmetric inactivation might simply reflect additive effects of inactivating one hemisphere in each region. Although unilateral inactivation of either pBLA or PL alone had no significant effects on the expression of learned fear, decreases in freezing behavior were observed in each case. It is possible that significant effects of unilateral pBLA and/or PL inactivation would have been observed had a weaker conditioning paradigm been used in the present study. Thus the impairments in fear memory expression induced by asymmetric or symmetric inactivation may have occurred independently of functional interactions between BLA and PL.

Another possibility is that more complex processing mechanisms within pBLA–PL circuitry are involved in fear memory expression. Equivalent impairments with asymmetric and symmetric BLA–PL inactivation on memory processing have been shown previously; this was interpreted as evidence for the involvement of parallel processing via intra-hemispheric and/or inter-hemispheric projections within the circuit (Fuchs et al., 2007). It is also possible that interactions between BLA, PL and other reciprocally connected regions are involved, such that processing mechanisms in a more extensive neural circuit are necessary for fear memory expression. One such region is hippocampus which plays a critical role in contextual fear memory processing (Anagnostaras

et al., 2001). The dorsal hippocampus is thought to encode contextual representations which may become associated with shock-related information in BLA, and possibly PL, via projections from ventral hippocampus (Maren & Fanselow, 1995; Pitkanen, Pikkarainen, Nurminen, & Ylinen, 2000; Takita, Izaki, Jay, Kaneko, & Suzuki, 1999; Vertes, 2004). Post-training lesions of ventral hippocampus impair the expression of contextual fear memory (Maren & Holt, 2004; Trivedi & Coover, 2004), although evidence from reversible inactivation and Fos expression studies render its precise role unclear (Albrechet-Souza, Borelli, Almada, & Brandão, 2011; Maren & Holt, 2004). Nevertheless, functional interactions between BLA, PL and hippocampus might be important for subserving contextual fear memory expression.

5. Conclusions

This study confirms previous evidence demonstrating the involvement of BLA and PL in mediating the expression of contextual fear memory and extends it by showing different roles for rostral and caudal BLA in this process. It also raises the possibility that BLA–PL circuitry is involved in subserving contextual fear memory expression via complex processing mechanisms, although further research is required to confirm the present results. These preliminary findings add to a growing body of evidence indicating that functional interactions between BLA and mPFC play an important role in fear memory processing. However, there are several unresolved issues that are worth considering here. Sodium channel inhibition was used to inactivate BLA and PL, thus it is possible that these findings are at least partly attributable to effects on local fibers of passages. Future studies using GABA receptor agonists should prove useful in clarifying this issue. As freezing was used to index fear, the present results may apply only to this but not other fear measures such as conditioned suppression of instrumental responding for reward. This is particularly relevant given the evidence that BLA is not required for fear-induced reductions in appetitive responding (Killcross, Robbins, & Everitt, 1997; Petrovich, Ross, Mody, Holland, & Gallagher, 2009). The present findings do not address whether the expression of learned fear and/or retrieval of fear memory were affected. Further experiments are needed to disentangle the roles of BLA, PL, and BLA–PL circuitry in these distinct processes. Future studies are also needed to determine if BLA–mPFC circuitry is similarly involved in the expression and/or retrieval of learned fear to explicit cues.

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